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Drivers of soil bacterial community structure and diversity in tropical agroforestry systems

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ABSTRACT

Agroforestry systems (AFSs) are considered sustainable forms of land management, but their impact on soil microbial biodiversity remains poorly understood. Here, we compared the structure and diversity of bacterial communities in rubber-based AFSs in tropical China that had different management types and stand ages: 10-year-old rubber mono- (YR) and intercropping (YRF), and 22-year-old rubber mono- (MR) and intercropping (MRF). Stand age had a stronger effect on bacterial communities than management type or season. Compared to the corresponding monocultures, YRF maintained bacterial diversity despite experiencing a decrease in abundance, especially at soil depths of 0–5 and 5–30 cm, whereas MRF enhanced bacterial diversity with a consistent abundance. Bacterial communities in AFSs exhibited obvious horizon-specific seasonal variations due to spatial and temporal heterogeneity in edaphic factors. *Acidobacteria*, *Proteobacteria* and *Actinobacteria* were the most dominant bacterial phyla in tropical AFSs in China, while *Kitasatospora*, *Streptomyces*, *Nitrospira*, *Bacillus* and *Pseudomonas* were the key genera. Soil pH, available phosphorus and dissolved organic nitrogen emerged as the major drivers of these bacterial community patterns. These findings indicate that the establishment of rubber-based AFSs, particularly those in mature stands, is a promising management practice for alleviating the adverse effects of rubber monoculture on bacterial biodiversity, including severe acidification and nutrient depletion. Our study highlights the importance of considering multiple effects when investigating bacterial communities in AFSs under climate change scenarios.

1. Introduction

Agroforestry systems (AFSs), in which at least one woody perennial species is grown alongside crops in order to increase diversity and productivity, are increasingly being recognized as a sustainable form of land use (Araujo et al., 2012). AFSs can provide multiple ecological benefits, including enhancement of carbon sequestration to mitigate climate change (Baah-Acheamfour et al., 2014), improvement in soil status (Liu et al., 2018) and diversification in soil microbial communities (Bagyaraj et al., 2015; Duchene et al., 2017). Soil microbes, particularly bacteria, are ubiquitous in forest ecosystems, where they play a critical role in ecosystem functions, such as the biogeochemical cycle and nutrient transformation for plant growth (Rodrigues et al., 2013). Nevertheless, the impacts of AFSs on the structure and diversity of soil bacteria are still poorly understood, and previous studies have reported either conflicting or inconclusive results (Lagerlöf et al., 2014; Banerjee et al., 2016; Wang et al., 2017; Berkelmann et al., 2018). Thus, a deeper understanding of how bacterial communities respond to AFSs, especially to different management regimes and edaphic gradients, is necessary for evaluating such ecosystem processes.

When establishing an AFS, it is important to consider stand age because this affects the growth of understory species by influencing light and water efficiency and thus litter quality and quantity (Peerawat et al., 2018). The distribution of roots and production of exudates can alter both soil pH and rhizosphere nutrients, and these processes vary with stand age due to competition or facilitation (Duchene et al., 2017; Zhang et al., 2017). Moreover, variations in carbon energy sources and soil pH have been shown to drive the community composition of some

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bacterial taxa (i.e. copiotrophs and oligotrophs) at different successional stages, such as the conversion tropical forests into rubber AFSs and intensive rubber monocultures (Fierer et al., 2007; Krashevska et al., 2015; Cai et al., 2018). Several studies have demonstrated the non-linear relationship between stand age and bacterial composition and diversity (Na et al., 2017; Zhou et al., 2017; Peerawat et al., 2018), emphasizing the importance of exploring the most appropriate stand age required to establish AFSs that can maintain ecosystem stability.

Indeed, bacterial communities experience pronounced temporal variability during seasonal and annual time scales in forests (López-Mondéjar et al., 2015; Žifčáková et al., 2016), croplands and grasslands (Cruz-Martínez et al., 2009; Lauber et al., 2013), thereby limiting our ability to identify the impacts of different management regimes on bacterial communities over the course of a growing season (Degrune et al., 2017). Bacterial communities in a temperate mountain forest are relatively stable across seasons, although fine-scale shifts may be concealed by considerable spatial variation (Kuffner et al., 2012). However, till date, most studies have been conducted in humid or semi-arid temperate regions, with little consideration of the dynamics of bacterial communities in the tropics, a region with distinct dry and wet seasons (Kivlin and Hawkes, 2016; Lan et al., 2017a, 2018; Monkai et al., 2018). Unfortunately, they also have focused exclusively on bacteria in the surface soil (reviewed by Fierer, 2017), despite the fact that key edaphic properties, such as soil pH and nutrients, often varying substantially with depth, resulting in marked shifts in bacterial communities along the soil profile (Eilers et al., 2012; López-Mondéjar et al., 2015; Engelhardt et al., 2018). Since all of these deficiencies are particularly true in terms of innovative AFSs, it is crucial to examine the seasonal effects on bacterial communities in tropical AFSs on the basis of vertical stratification.

Unprecedented large-scale conversion of tropical forests to rubber monocultures to meet worldwide demand for consumer products has caused deforestation and a range of ecological and social impacts (Liu et al., 2017). As a result, rubber monoculture is widely believed to be one of the main causes of biodiversity loss, although some studies have found that this is not necessarily the case for bacteria, with comparable (Berkelmann et al., 2018; Cai et al., 2018) or higher bacterial diversity occasionally observed in monocultures than in forests (Kerfahi et al., 2016; Lan et al., 2017a, b). Diverse rubber-based AFSs, particularly those in which nitrogen-fixing species (NFSs) are mixed, have been proposed as a method for generating additional income and sustaining soil fertility without affecting rubber yield (Jessy et al., 2017). Introduced perennial NFS (i.e., shrub and tree) can modify soil parameters and consequently influence belowground microbial community structure, with specific plant species associating with unique microbial assemblages (Bagyaraj et al., 2015; Kivlin and Hawkes, 2016; Duchene et al., 2017), although some bacterial taxa: *Acidobacteria*, *Proteobacteria* and *Actinobacteria* are prevailing in these AFSs. For instance, addition of an NFS to Eucalyptus plantations resulted in a high bacterial abundance and diversity (Huang et al., 2014). However, our current understanding of bacterial characteristics associated with NFSs that may affect rubber management is limited, and there appears to be no consistent trend in bacterial diversity because it is closely correlated with rubber stand age (Peerawat et al., 2018). Furthermore, no study has attempted to determine the temporal response of bacterial communities to rubber-based AFSs.

The aim of this study was to explore the seasonal dynamics of bacterial communities in rubber-based AFSs under two different management types (monocropping and intercropping) and two different stand ages (10 and 22 years old, representing young and mature stands, respectively). This was achieved by examining bacterial communities and the relevant plant and soil physicochemical properties at three depths depending on the vertical distribution of fine roots. We hypothesized that i) seasonality would strongly drive differences in bacterial communities due to temporal changes in plant–soil interactions; ii) rubber-based AFSs would serve to enhance bacterial abundance and diversity across seasons; iii) bacterial communities would be vertically stratified, with an increased abundance and diversity in the surface soils; and iv) the key taxa would vary with management type and stand age, but the factors modulating bacterial communities would be relatively consistent.

2. Materials and methods

2.1. Field site description

This study was conducted in environment-friendly rubber plantations at Xishuangbanna Tropical Botany Garden, Chinese Academy of Sciences, Mengla County, Southwestern China (21°33′N, 101°28′E; 880 m asl.). This site lies on a natural slope (15° to 25°) and has an altitudinal range of 870–890 m, with soil characterized as Oxisol, as per the USDA Soil Taxonomy guidelines. This site has a typical tropical monsoon climate, with distinct dry (dry-hot and fog-cool) and wet (rainy) seasons, with a mean annual precipitation and air temperature of 1480 mm and 22 °C, respectively. During the experimental period (January to December 2016), the mean annual temperature was 22 °C, with a monthly maximum of 26 °C in June and a monthly minimum of 15 °C in January. The site received 1295 mm of annual rainfall. The dry-hot season started at the end of February and continued until mid-May, whereas the rainy season continued from May to October, during which approximately 1087 mm of rainfall was recorded, accounting for 84% of the total annual rainfall. The fog-cool season spanned the remainder of the year, during which there were only five rainfall events > 10 mm.

2.2. Experimental design and sampling

Prior to the establishment of the environment-friendly rubber plantations, the study site was dominated by rubber monocultures planted in 1994 and 2006 with a conventional 2.5 m × 8 m spacing arrangement. In June 2010, seven lines of the native NFS *Flemingia macrophylla*, a species well adapted to tropical climate and local soil conditions, were interplanted in a section of each monoculture stand at a density of 0.8 m × 1.0 m inter-row spacing. All sites were managed in a similar way, with the exception that *F. macrophylla* was clipped approximately 30 cm above the ground and subsequently covered in situ at the end of each year. In March 2016, three sample plots of 20 m × 25 m were randomly delineated in each of the four stands, namely the 10-year-old rubber monocropping (YR), 10-year-old rubber intercropping (YRF), 22-year-old rubber monocropping (MR) and 22-year-old rubber intercropping (MRF) (Fig. 1). Variation in topography and spatial representation were taken into account, and two adjacent plots within the same experimental stand were separated by a buffer zone. Descriptions of the stand characteristics are provided in Table 1.

Soil samples were obtained from three optimal locations in each plot: i.e., the rubber rhizome area, the ruderal zone and the area around the fertilization zone (Liu et al., 2018), during the dry-hot (30 March), rainy (20 August) and fog-cool (30 December) seasons. At each location, the samples were collected from depths of 0–5, 5–30 and 30–60 cm (after removing the litter layer) using a 5-cm auger. Samples were then homogenized and subdivided according to plot and depth. Part of each sample was frozen at –80 °C for molecular analysis, whereas the remainder was stored at 4 °C for the analysis of physicochemical properties. All fine roots that occurred in cores of the same depth were also collected. Meanwhile, the litter layer was collected every time from a 50 cm × 50 cm square close to each soil core. Fine roots and litter layer materials were then oven-dried at 70 °C to obtain a constant weight and finely ground for total element analysis. The stocks of the fine roots and litter layer were calculated by multiplying the respective C, N, P concentrations by dry mass and were represented by the sum of the three sampling times.

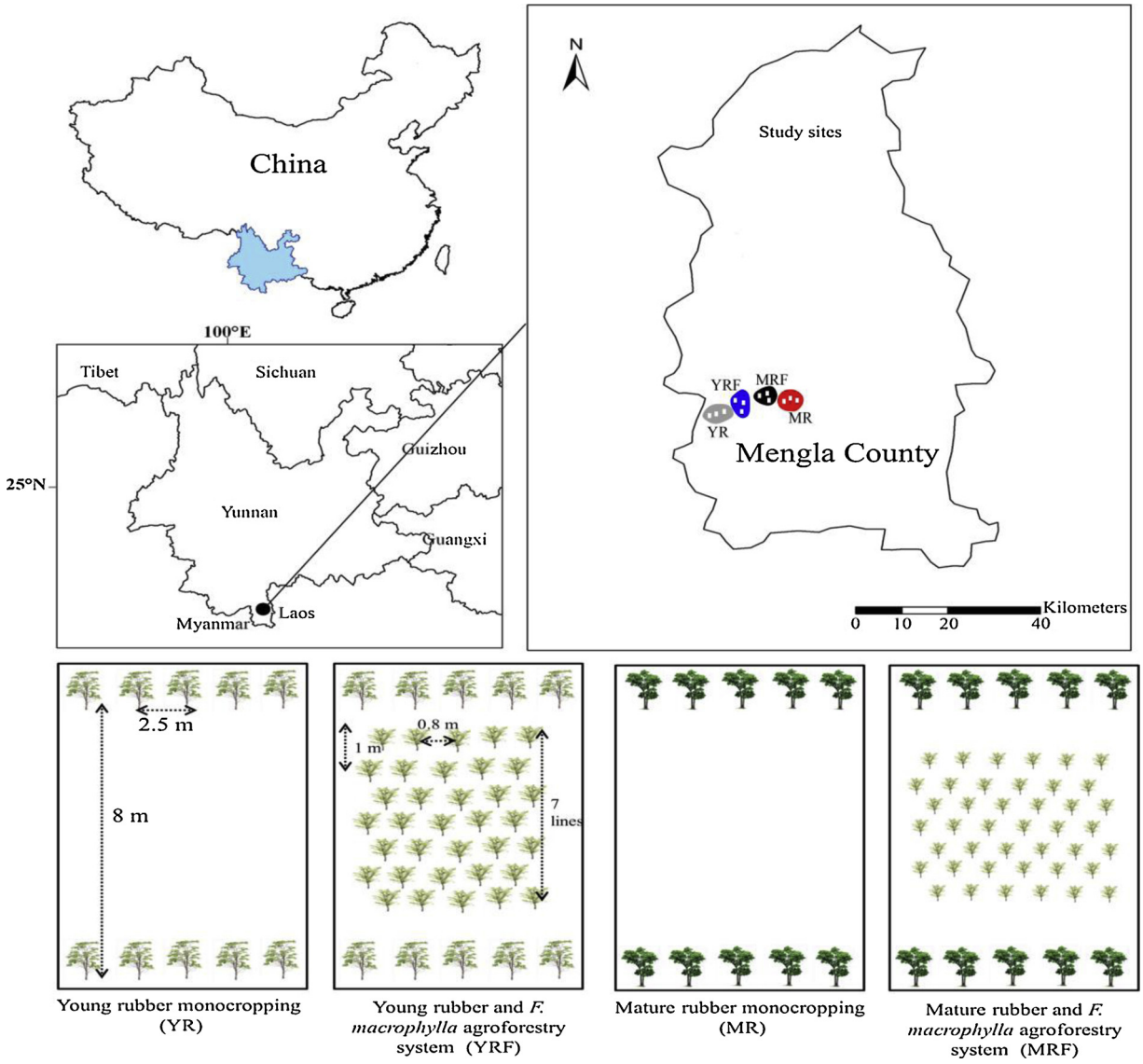

Fig. 1. The sketch map of rubber-based agroforestry systems.

2.3. Physicochemical analyses of soils and plants

The gravimetric soil moisture content of each soil sample was determined by oven-drying a 20-g subsample at 105 °C to obtain a constant weight. Soil temperature was measured in situ for three consecutive days using a geothermometer. Soil pH was measured in a 1:2.5 mixture of soil to water using a digital pH meter (FE28, Mettler Toledo, USA). Total C (TC) and total N (TN) were analyzed with a CN analyzer (Vario MAX CN; Elementar, Germany). Total P was digested using HNO_3–$HClO_4$ and determined by ICP-AES (iCAP6300, Thermo Fisher Scientific, USA). NO_3^--N, ammonium-N (NH_4^+-N) and dissolved total N (DTN) were extracted from 10 g of fresh soil with 2 M KCl and analyzed using a segmented flow analyzer (Auto Analyzer 3; SEAL Analytical GmbH, Germany). Dissolved organic N (DON) was calculated according to the equation DON = DTN – NO_3^--N – NH_4^+-N. Dissolved organic C (DOC) was extracted from 10 g of fresh soil with deionized water and determined using a TOC analyzer (Vario TOC cube; Elementar, Germany). Available phosphorus (AP) was extracted from 5 g of air-dried soil with 0.03 M NH_4F + 0.025 M HCl and analyzed using a segmented flow analyzer. Microbial biomass C (MBC) and N (MBN) were extracted from 20 g of fresh soil using 0.5 M K_2SO_4 extraction procedure, and their concentrations were estimated based on the difference between the chloroform fumigated and unfumigated soil divided by correction factors of 0.45 and 0.54, respectively. Microbial biomass P (MBP) was extracted with 0.5 M $NaHCO_3$, and its concentration was estimated as the difference between the fumigated and unfumigated soil divided by a correction factor of 0.4.

2.4. DNA extraction

Total DNA was extracted from 0.25 g fresh soil using a PowerSoil isolation kit (MoBio, Carlsbad, USA) following the manufacturer's instructions. DNA concentration and purification were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA), and its quality was checked by 1.2% agarose gels. Extracted DNA was diluted in DES buffer and stored at −80 °C for further analysis.

2.5. Quantitative real-time PCR (qPCR)

The bacterial abundance of soil sample was quantified in triplicate by targeting bacterial 16S rRNA gene using qPCR in a FTC-3000™ (Funglyn, Shanghai, China) with the primer set 515 F (5′-GTGCCAGC MGCCGCGGTAA-3′) and 926R (5′−CCGTCAATTCMTTTGAGTTT-3′) following the protocol reported previously (Ochoa-Hueso et al., 2015).

Table 1
Stand characteristics of the four experimental sites. Data followed by different letters differ significantly among stands according to the Tukey's test ($P < 0.05$). DB – mean of diameter at the base, DBH – mean of diameter at the breast height. The latter of slash represents base diameter and height of *F. macrophylla*.

Stand type	DBH/DB (cm)	Mean height (m)	Basal area (m^2 ha^{-1})	Litter layer (t ha^{-1} yr^{-1})	C in litter layer (t ha^{-1} yr^{-1})	N in litter layer (kg ha^{-1} yr^{-1})	P in litter layer (kg ha^{-1} yr^{-1})	Fine roots (0–60 cm) (kg m^{-3} yr^{-1})	C in fine roots (kg m^3 yr^{-1})	N in fine roots (g m^{-3} yr^{-1})	P in fine roots (g m^{-3} yr^{-1})
YR	14.4(0.5)b	8.7(0.2)b	8.4 (0.5)b	8.3(0.8)c	2.8(0.3)c	87.7(9.0)c	5.8(0.2)c	4.3(0.1)b	1.2(0.0)b	33.6(0.4)b	7.1(0.1)a
YRF	14.9(0.3)b/2.2 (0.1)a	8.2(0.1)b/2.9(0.1)a	8.6(0.3) b	22.4(0.1)a	7.3(0.7)a	339.7(43.5)a	20.1(1.9)a	5.5(0.5)ab	1.4(0.1)b	51.2(4.5)a	8.2(0.7)a
MR	20.1(0.6)a	15.5(0.1)a	14.5(0.8)a	9.9(0.8)c	3.5(0.4)c	119.9(11.3)c	6.1(0.4)c	6.9(0.3)a	2.0(0.1)a	54.4(2.6)a	7.3(0.3)a
MRF	21.5(0.3)a/1.3(0.1)b	16.0(0.1)a/1.1(0.1)b	16.1(0.5)a	16.4(1.7)b	5.2(0.4)b	232.6(25.2)b	11.5(1.9)b	5.3(0.2)b	1.6(0.1)b	47.1(1.8)a	4.4(0.2)b

Briefly, PCR reaction mixture (25 μL) contained 12.5 μL of 2 × SYBR Premix Ex Taq™ (Takara, Japan), 1 μL of each forward and reverse primers (10 μM), 5 μL of template DNA and sterilized deionized water. Thermal program was initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. Negative controls consisted of all reagents with sterilized water instead of soil DNA. The abundance of bacteria was calculated using a linear regression equation to convert the cycle threshold value to the known number of copies in the standard curves.

2.6. Illumina MiSeq sequencing

The V4–V5 region of bacterial 16S rRNA genes was amplified using the universal primers 515 F/926R, these primers were chosen because of their high coverage of almost all phyla in conventional and metagenomic studies (Rodrigues et al., 2013). The primers also contained the lllumina 5′ overhang adapters for two-step amplicon library building, following manufacturer's instructions for the overhang sequences. Amplification reactions were performed in two steps as previously described (Baldrian et al., 2012). In the first step, each of three independent 50-μL reactions per DNA sample contained 10 μL of 5 × buffer, 1 μL of dNTPs (10 mM), 1 U of Phusion DNA polymerase, 1 μL of each inner primer (10 μM) and 40 ng of template DNA. The amplification was initiated with 2 min at 94 °C, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. All PCR products were pooled and purified using agarose gel electrophoresis followed by the AxyPrep purification kit (Axygen Biosciences, Union City, USA). Products were used as the template for a second round of PCR using a composite primer containing multiplex identifiers. The second PCR was performed in a 40-μL volume containing 8 μL of 5 × buffer, 1 μL of dNTPs (10 mM), 0.8 U of Phusion DNA polymerase, 1 μL of each outer primer (10 μM) and 5 μL of template. Amplification was started with 2 min at 94 °C, 8 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. Prior to library pooling, the barcoded PCR products were purified using the AxyPrep purification kit. Sequencing was conducted on an Illumina MiSeq paired-end (2 × 300 bp) platform with the reagent kit version 3 at TinyGene Biotech (Shanghai) Co., Ltd. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database under accession number PRJNA508602.

2.7. Processing of pyrosequencing data

All raw sequences were processed using the bioinformatics platform mothur v. 1.33.3. Briefly, pyrosequencing data were demultiplexed by barcode and filtered to remove any ambiguous and homologous bases that were < 200 bp or > 580 bp in length or had an average quality score of < 20 over a 10-bp sliding window. Chimeric sequences were identified and eliminated using the UCHIME algorithm. Sequences that passed these quality filters were aligned and classified using the SILVA reference database. Any sequences that aligned to chloroplast sequences or were unknown at the domain level were discarded from the downstream analysis (de Carvalho et al., 2016). Pairwise distances between sequences were calculated using the farthest neighbour algorithm, and operational taxonomic units (OTUs) were constructed at a 97% similarity threshold. OTUs were then assigned to taxonomic groups using a randomly selected representative sequence with the classifier platform within the Ribosomal Database Project.

2.8. Statistical analyses

Alpha diversity was measured as species richness, evenness, Shannon–Wiener index (in which the relative abundance of species is weighted by evenness) and Faith's phylogenetic diversity (PD, which

sums the branch lengths of a phylogeny for a given plot and uses the result as a metric of phylogenetic diversity). The alpha diversity of bacterial communities among all treatments was compared by randomly selecting 20,540 sequences from each sample to ensure that the same level of survey effort was used. Differences in the plant properties, soil characteristics, alpha diversity and relative abundance of individual bacterial taxa among stands at each sampling time were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference test. The single and interactive effects of age and management on various soil physicochemical characteristics and alpha diversity of the same depth were tested using two-way repeated-measures ANOVA, with sampling time (dry-hot, rainy and fog-cool seasons) as the repeated measure.

The significance of observed differences in bacterial communities among the management type, stand age or season was determined by permutational multivariate ANOVA with Bray-Curtis similarity matrices after 999 permutations. Mantel test were calculated using the microbial dissimilarity matrix and environmental dissimilarity matrix, where microbial dissimilarity was calculated based on the Bray-Curtis method using the OTU table and environmental dissimilarity was calculated based on the Euclidean method using the soil variables. Non-metric multidimensional scaling (NMDS) ordination plot was constructed based on the UniFrac distance, and redundancy analysis (RDA) was conducted to visualize the relationships between normalized OTU abundances and environmental variables. The response ratio was calculated to illustrate changes in the relative abundance of bacteria compared with the same-aged stand under monocropping with a 95% confidence interval (Zeng et al., 2016). Analyses were performed using SPSS v.17.0, Canoco v.5.0 and the *vegan* and *sem* packages in R v.3.4.2 ($P < 0.05$).

3. Results

3.1. Soil characteristics and pyrosequencing dataset analysis

Soil properties were affected by stand age, management type and sampling season at all three depths, with season, in particular, causing significant differences in physicochemistry (Table S1). Across all seasons, soil temperature was lower, while NO_3^--N and AP concentrations were higher in YRF than in YR at 60 cm depth (Table 2 and Fig. S1). Similarly, soil temperature was lower while SWC, pH, NO_3^--N, NH_4^+-N and MBN were higher in MRF than in MR at 60 cm depth. SWC, MBC, DON and MBN increased with stand age, whereas pH, NO_3^--N and AP decreased. The physicochemical properties also showed significant changes with soil depth, with C/N ratio, N forms, microbial biomass and AP, in particular, decreasing with depth.

A total of 3,671,021 raw sequences were obtained from the 108 samples collected. 59.4% of which remained for analysis after filtering, denoising and the removal of chimeric and short sequences. An average of 20,203 classifiable sequences was obtained per sample (minimum 14,185). All of these sequences were clustered into 7156 OTUs at a 97% similarity threshold.

3.2. Bacterial community composition

The overall bacterial communities were dominated by *Acidobacteria* (28.0%), *Proteobacteria* (26.6%), *Actinobacteria* (13.4%) and *Chloroflexi* (10.9%) (Fig. 2 and Table S2). Within *Proteobacteria*, the dominant classes were *Alphaproteobacteria* (9.0%), *Betaproteobacteria* (7.5%), *Deltaproteobacteria* (6.9%) and *Gammaproteobacteria* (3.3%). Other less-abundant phyla (relative abundance 0.5%–5%) included *Planctomycetes*, *Nitrospirae*, *Gemmatimonadetes*, *Bacteroidetes*, *Firmicutes*, *Latescibacteria*, *Verrucomicrobia* and *Armatimonadetes*.

Bacterial composition was significantly affected by management type, stand age and season at all three soil depths ($P < 0.001$), with no significant interaction between these factors ($P > 0.05$). The relative

Table 2
Soil physicochemical properties of the four experimental sites at the depths of 0–5, 5–30 and 30–60 cm. Data are means of 9 replicates from three seasons with standard errors (in brackets). Unit of all other soil properties (with exception of soil temperature °C, SWC% and pH) are mg kg^{-1} soil. SWC – soil water content, C/N ratio – carbon: nitrogen ratio, DOC – dissolved organic C, MBC – microbial biomass C, NO_3^--N nitrite, NH_4^+-N ammonium, DON – dissolved organic N, MBN – microbial biomass N, AP – available phosphorus, MBP – microbial biomass P.

Soil properties	0–5 cm				5–30 cm				30–60 cm			
	YR	YRF	MR	MRF	YR	YRF	MR	MRF	YR	YRF	MR	MRF
Temperature	22.4(1.4)	21.5(1.4)	22.0(1.5)	21.2(1.3)	21.7(1.1)	20.8(1.1)	21.4(1.2)	20.6(1.1)	20.9(0.7)	19.6(0.6)	20.5(0.8)	19.4(0.6)
SWC	19.4(2.6)	24.0(1.9)	25.5(2.3)	26.9(2.2)	19.5(1.4)	19.9(1.3)	22.9(1.6)	23.4(1.4)	22.1(1.2)	20.6(1.2)	23.4(1.2)	25.8(1.2)
pH	6.3(0.1)	6.2(0.1)	5.3(0.1)	5.6(0.1)	6.2(0.1)	6.3(0.1)	5.2(0.1)	5.5(0.1)	6.1(0.1)	6.4(0.1)	5.3(0.1)	5.4(0.1)
C/N ratio	8.8(0.1)	9.1(0.2)	9.5(0.1)	9.0(0.1)	7.8(0.1)	8.2(0.2)	8.5(0.2)	8.1(0.1)	6.8(0.2)	6.8(0.2)	6.4(0.1)	6.7(0.2)
DOC	79.3(6.2)	87.2(6.3)	79.5(3.5)	86.1(3.7)	67.6(7.2)	68.0(7.2)	66.0(3.8)	74.6(7.4)	65.0(8.3)	61.0(7.7)	61.3(6.7)	69.1(6.6)
MBC	284.1(29.1)	362.3(58.4)	341.9(68.5)	412.2(43.2)	157.6(32.4)	146.8(30.6)	301.1(74.7)	209.0(11.3)	129.7(32.2)	175.9(31.5)	202.5(67.9)	213.5(23.7)
NO_3^--N	3.0(0.5)	3.5(0.5)	0.5(0.1)	3.1(0.6)	1.1(0.3)	2.4(0.4)	0.8(0.1)	1.6(0.2)	0.9(0.2)	1.6(0.3)	0.6(0.1)	0.5(0.1)
NH_4^+-N	6.7(1.8)	9.4(2.1)	6.2(1.3)	9.0(1.9)	4.7(0.8)	4.5(1.0)	5.4(0.9)	6.8(1.4)	4.1(0.9)	3.1(0.5)	3.4(0.6)	4.6(0.9)
DON	40.2(3.8)	40.9(3.6)	54.6(2.6)	44.7(3.3)	45.7(3.8)	44.1(3.7)	53.0(3.1)	48.1(2.2)	45.7(3.4)	44.4(3.3)	56.6(2.6)	55.7(2.7)
MBN	48.5(7.1)	61.7(8.1)	57.9(9.5)	83.2(7.6)	32.5(6.2)	28.5(4.9)	34.3(8.5)	53.8(9.6)	18.0(3.7)	20.8(4.0)	21.8(6.7)	37.5(8.7)
AP	2.2(0.1)	6.1(0.5)	1.4(0.1)	1.4(0.1)	1.3(0.1)	3.5(0.3)	0.8(0.1)	0.7(0.1)	0.7(0.1)	2.1(0.3)	0.4(0.2)	0.2(0.1)
MBP	4.5(0.5)	5.2(0.8)	3.5(0.5)	4.7(0.3)	1.5(0.3)	2.1(0.4)	1.5(0.4)	2.1(0.5)	0.8(0.2)	1.1(0.3)	1.0(0.3)	1.0(0.3)

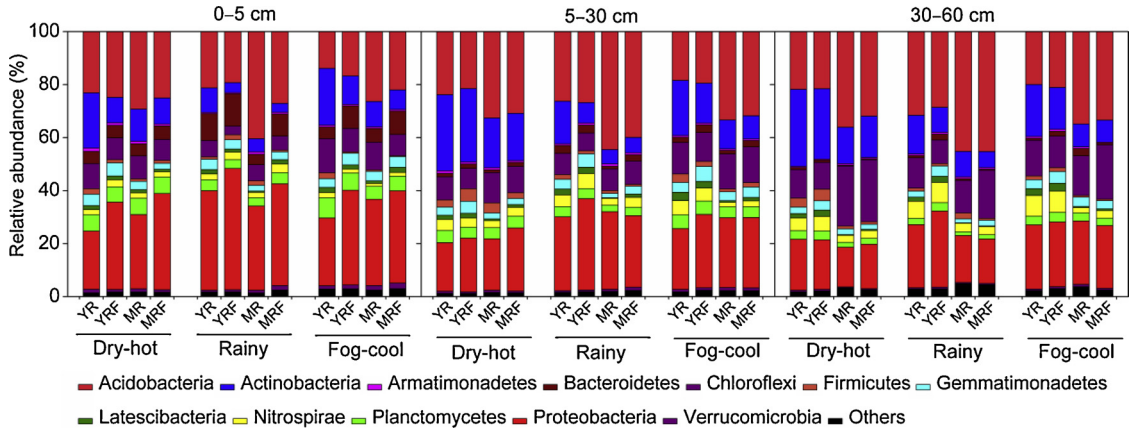

Fig. 2. Bacterial community composition at the phylum level (> 0.5%) in rubber-based agroforestry systems at soil depths of 0–5, 5–30 and 30–60 cm during three sampling seasons. The data represent the means of three replicates per season.

abundance of *Actinobacteria* and *Chloroflexi* was lower in YRF than in YR across seasons, particularly at 0—5 cm depth, whereas *Gemmatimonadetes* and *Proteobacteria* displayed an opposite trend (Fig. 2). Similarly, *Acidobacteria* had a lower relative abundance in MRF than in MR at 0–5 cm depth, whereas *Proteobacteria* exhibited an opposite trend. *Nitrospirae* and *Chloroflexi* had higher relative abundance in MRF than in MR at depths of 5–30 and 30–60 cm, respectively.

A strong shift in bacterial relative abundance was observed at 0–5 cm depth in YRF, with 6 phylotypes (from phylum to family) exhibiting changes in the dry-hot season, 6 in the rainy season and 8 in the fog-cool season. By contrast, 1, 13 and 7 families exhibited significant changes in the same soil layer in MRF across these three seasons, respectively (Table S3). Moreover, an average of 3 and 4 phylotypes at depths of 5–30 and 30–60 cm, respectively, exhibited obvious changes in relative abundance in YRF across seasons, whereas only 2 families were affected at 5–30 cm and no family was affected at 30–60 cm in MRF. These dynamic responses at the three soil depths were further supported by the response ratio, which showed that most of the affected taxa were relatively rare (abundance lower than the average median value of 1.4%); these taxa exhibited increasing or decreasing trends with different management types and seasons (Fig. 3). Therefore, the response of bacterial groups evidently depended on the abundance of lower-level taxa. However, some minor changes in lower-level taxa were not observed at the phylum level due to their low abundance. For instance, changes in *Nitrospiraceae* in YRF at 30–60 cm depth and *Acidothermaceae* in MRF at 0–5 cm did not lead to corresponding changes in *Nitrospirae* and *Actinobacteria*, respectively.

In total, 402 bacterial genera were identified in this study (data not shown). The most abundant genera, including *Kitasatospora*, *Streptomyces* (*Actinobacteria*) and *Roseiflexus* (*Chloroflexi*), were lower in YRF than in YR at all three soil depths across seasons. However, the main actinobacterial genera *Kitasatospora* and *Acidothermus* were present at similar abundances in MRF and MR.

3.3. Bacterial community diversity

Almost all diversity parameters were affected by stand age, management type and season at all three soil depths (repeated-measure,

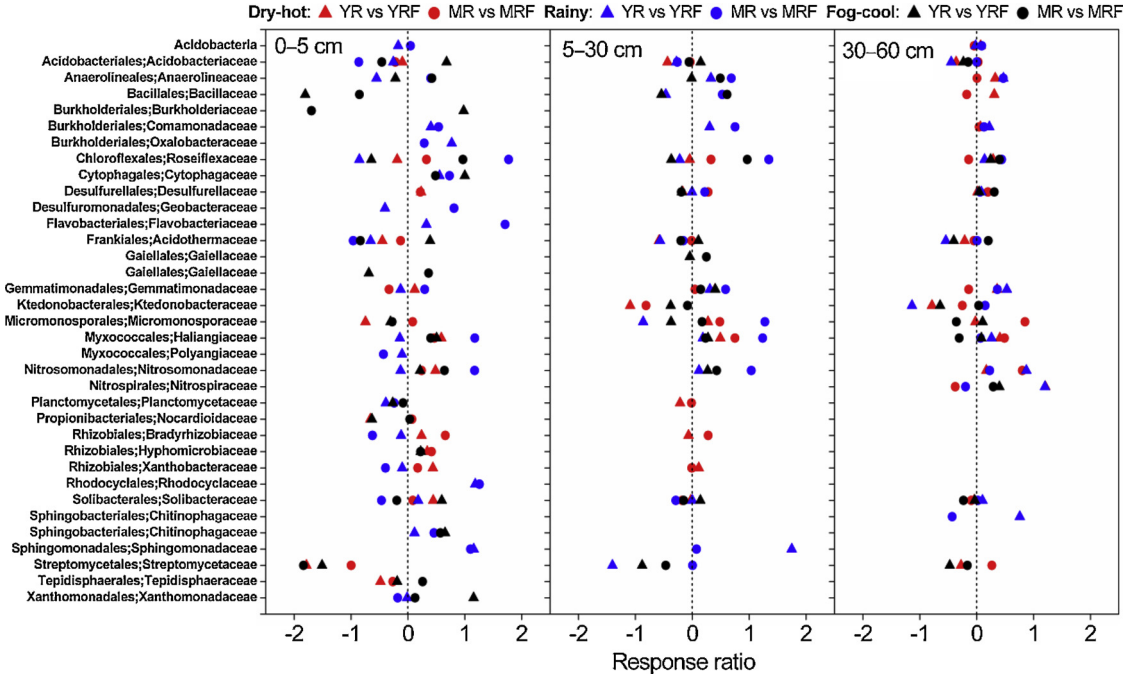

Fig. 3. Significantly altered bacterial taxa (> 1%) at the lowest taxonomic level following management and season at soil depths of 0–5, 5–30 and 30–60 cm, as measured by the response ratio method with a 95% confidence interval.

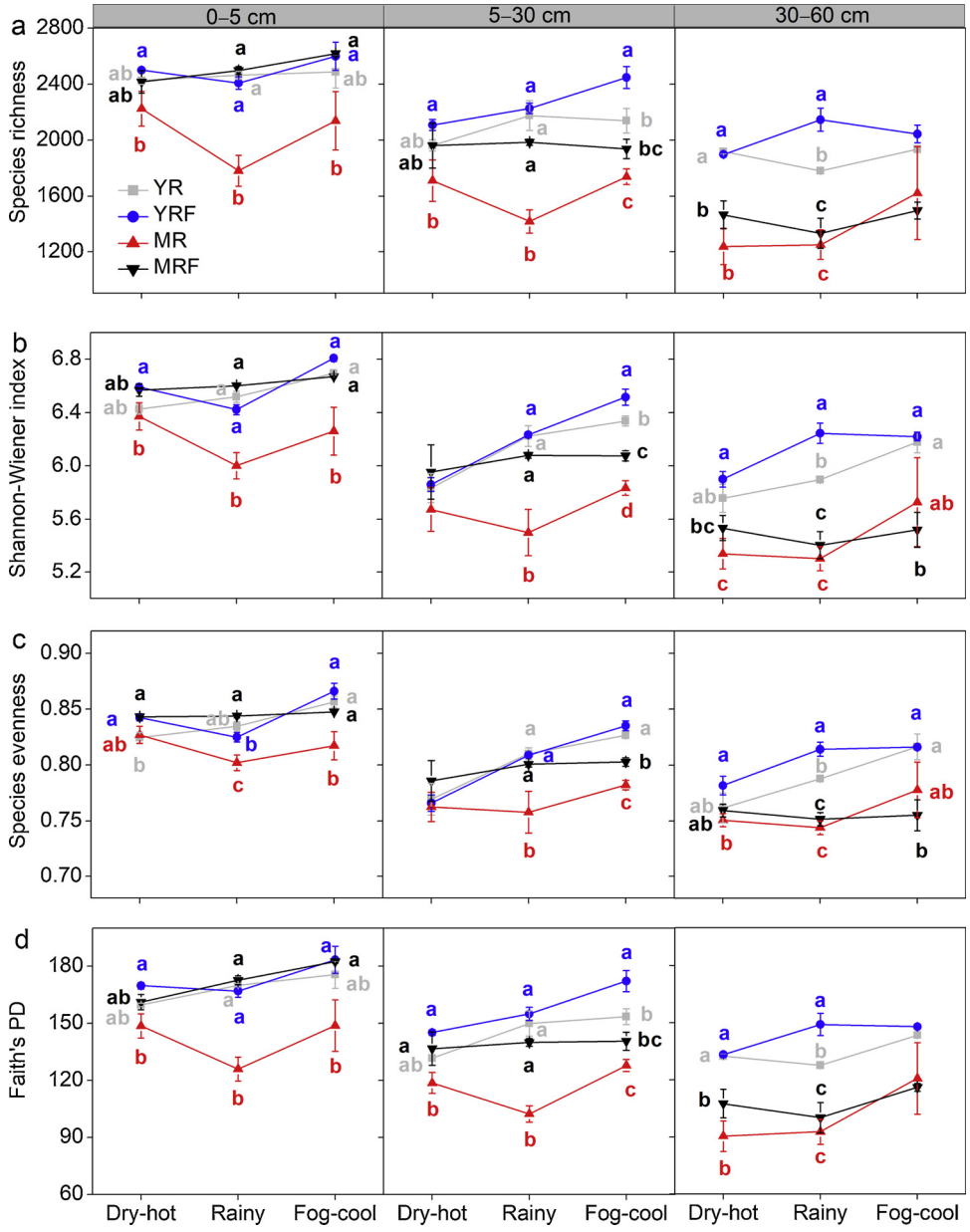

Fig. 4. Bacterial diversity indices of rubber-based agroforestry systems at soil depths of 0–5, 5–30 and 30–60 cm during three sampling seasons. The data represent the means of three replicates with standard errors. Different letters indicate statistically significant differences among management types in each season ($P < 0.05$).

$P <$ 0.05), with the highest values being observed in the fog-cool season. Species richness, diversity (expressed as the Shannon–Wiener index) and Faith's PD were higher in YRF than in YR at 5–30 cm depth in the fog-cool season and at 30–60 cm in the rainy season (Fig. 4a, b and d), whereas bacterial abundance and diversity were higher in MRF than in MR at depths of 0–5 and 5–30 cm in the rainy and fog-cool seasons. Species evenness was also greater in YRF than in YR at 0–5 cm depth in the dry-hot season and at 30–60 cm in the rainy season, whereas MRF had a more homogeneous community than MR at depths of 0–5 cm and 5–30 cm in the rainy and fog-cool seasons (Fig. 4c). All diversity metrics decreased with increasing soil depth irrespective of management type and season. Bacterial diversity was strongly correlated with pH, AP, DON and NO_3^--N. However, these correlations varied with season and soil depth (Table S4), being particularly strong in the rainy season.

3.4. Bacterial community structure

The NMDS plots revealed that soils from the two different-aged stands harbored distinct bacterial communities at the three soil depths regardless of management type and season (Fig. 5a). The ADONIS analysis further confirmed that bacterial communities in young AFSs were significantly different from those in mature ones (Fig. S2). There was also a clear bacterial community differentiation between the different seasons or management types (Fig. S3). The RDA plots showed that the relationships between physicochemical variables and bacterial composition at each soil depth differed across the three seasons (Fig. 5b and Fig. S4). The result of Mantel test showed that pH, AP and DON had the largest overall effect on community structure (Table S5).

4. Discussion

This study represents a first attempt to systematically assess the effects of AFSs on bacterial communities in tropical China. It was

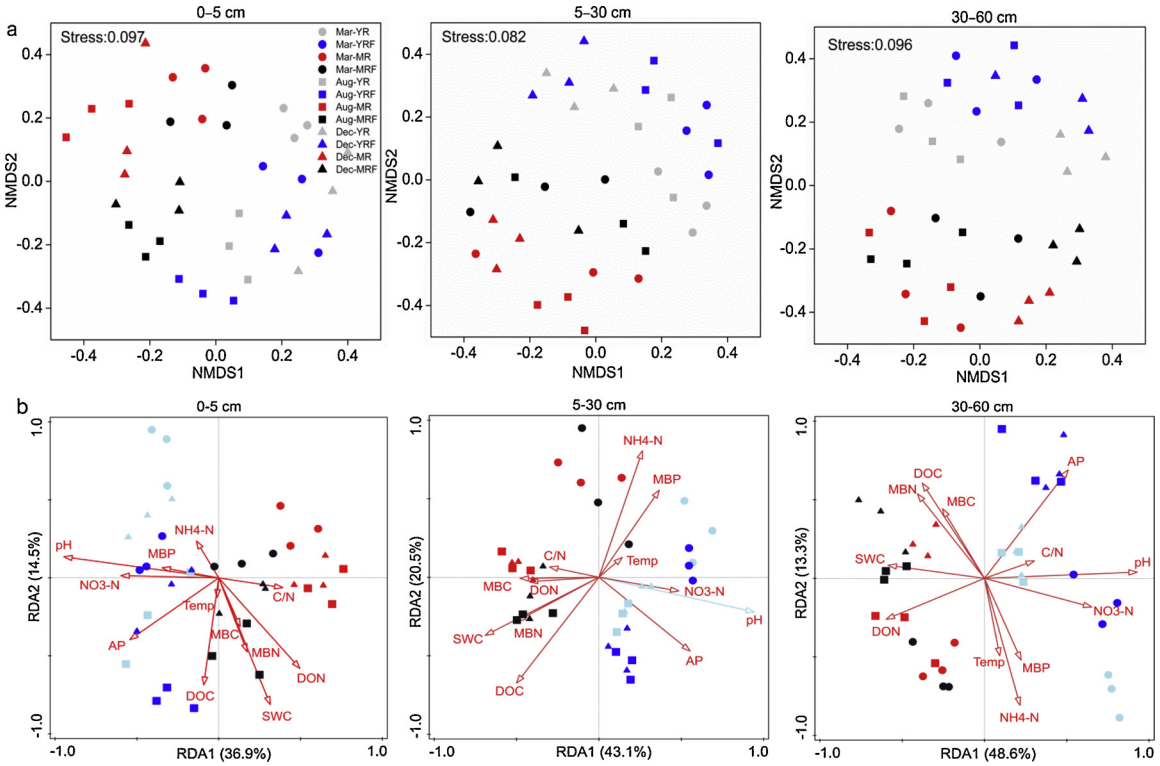

Fig. 5. Nonmetric multidimensional scaling (NMDS) plot of soil bacterial communities and redundancy analysis (RDA) of changes in bacterial communities with environmental variables based on the number of OTUs in rubber-based agroforestry systems at soil depths of 0–5, 5–30 and 30–60 cm during three sampling seasons.

conducted in two different-aged rubber AFSs and their associated monoculture control plots located within a micro-region with similar land-use history to avoid confounding effect in topography. In addition, changes in bacterial communities were also evaluated at three soil depths during a year. This stratified and temporal sampling potentially captures the underlying biophysical differences in soil horizons, plant roots and climatic conditions characteristics of most terrestrial tropical ecosystems, allowing the extrapolation of observed differences in bacterial community structure and diversity to areas beyond this site, and providing a more comprehensive understanding of AFS establishment and management strategies.

4.1. Stand age has a stronger effect on bacterial communities than management type or season

Bacterial communities in two different-aged rubber plantations were clearly differentiated in the NMDS plots, with stand age demonstrating 31%–48% of the total variability with depth (Fig. S2). When rubber trees reached maturity, the relative abundances of oligotrophs (e.g. *Acidobacteria* and *Chloroflexi*) increased at all soil depths in parallel with decline in copiotrophs (e.g. *Gemmatimonadetes* and *Bacteriodetes*) and *Actinobacteria*, irrespective of the NFS introduction. Bacterial diversity was also lower in mature stands than in young ones, with the greatest difference observed at 30–60 cm (Fig. 4). Notably, these changes were mainly attributed to MR, wherein long-term latex collection causes severe soil acidification and nutrient depletion, particularly of P (Liu et al., 2018). Similarly, Peerawat et al. (2018) found that mature rubber plantations with no tillage and crop harvesting can harbor specific, but less diverse, bacterial communities dominated by *Firmicutes*, indicating a stable and undisturbed environment. Although latex tapping reduces soil fertility with increasing rubber age, bacterial diversity initially increases due to a complex succession of understory vegetation till the mature stage and then decreases in the old phase (Zhou et al., 2017). These changes imply that stand age has a non-linear effect on bacterial diversity, with a specific taxon composition being present during the entire lifespan, which largely depends on management intensity and original land-use types.

Season explained 11%–17% of the total variability with depth, indicating that seasonality had an important effect on soil bacterial communities in these AFSs. Possible explanations for the effect of seasonality on bacterial communities vary among previous studies. Fluctuations in soil moisture and temperature are considered the most likely causes of compositional shifts in bacterial communities (Rasche et al., 2011). However, previous studies on bacterial community structure revealed only subtle seasonal changes in response to moisture and temperature (Kuffner et al., 2012; Lan et al., 2018). Bacterial communities in rubber plantations have been shown to be affected by seasonal changes in nutrient levels (Lan et al., 2017a), and this seems to be a plausible explanation for the observed differences in community compositions in the fog-cool season (Fig. 5), during which time, *Proteobacteria* had the highest relative abundance. In the fog-cool season, labile P and microbial biomass reached maxima due to a seasonal peak in photosynthate allocation to the roots and rhizodeposition and soils also showed the highest bacterial diversity (Fig. 4). This contrasts with another tropical study that diversity peaked in the dry-rainy season (Lan et al., 2018), indicating that different bacterial communities are sensitive to different seasons of the year. Thus, seasonal variability in bacterial community needs to be carefully assessed across different land management types, even for soils exposed to the same climatic conditions.

Management type had a relatively small effect on bacterial communities, explaining only 8%–3% of the total variability with depth. This is consistent with a previous finding that treatment effects did not exceed seasonal variability in a Californian grassland (Cruz-Martínez et al., 2009), but contrasts with the results of Lauber et al. (2013) who found that bacterial compositional differences across land management types were more pronounced than temporal dynamics due to high relative abundances of certain taxa. Regardless of stand age and season, rubber-based AFSs had a large number of copiotrophs, particularly *Proteobacteria* and *Bacteroidetes* at 0–30 cm depth (Fig. 2), which can be

attributed to a general improvement in soil quality. In addition, the AFSs, particularly MRF, hosted a high bacterial diversity. The introduction of an NFS will have reduced the problem of intensive management common in rubber monocultures, resulting in a less-disturbed environment. Our results support the conclusion that bacterial diversity is high under moderate land management intensity (Peerawat et al., 2018) and suggest that although land management only explains a tiny proportion of the overall variation, its importance for bacterial communities cannot be ignored when comparing AFSs with monocrop systems.

4.2. Mature rubber-based AFS maintains bacterial abundance and enhances diversity

MRF soil maintained stable bacterial abundance relative to MR (Fig. S5), which is in line with the finding that agroforestry practices (15–30 years) were not sufficient to alter bacterial abundance (Lagerlöf et al., 2014). However, Canadian AFSs tended to show higher bacterial abundance than adjacent cropland due to the nutrient-rich soil resources (Banerjee et al., 2016). MRF had a greater bacterial diversity than MR (Fig. 4), supporting the positive effect of mixing an NFS into *Eucalyptus urophylla* plantation (Huang et al., 2014). A recent study showed the negative effect, with agroforestry soils harboring lower bacterial diversity and constant richness, suggesting the presence of many rare species (Banerjee et al., 2016). This discrepancy is mainly attributed to differences in soil pH that is known to be a major determinant of bacterial diversity (Lauber et al., 2009). Increased pH is a key factor that favors nutrient capture in rubber production systems on highly acidic soils. The significant positive relationship we observed between increased bacterial diversity and soil pH can be corroborated by a pH gradient experiment (Rousk et al., 2010), which showed that the slope of this relationship was the highest below a pH of 5.4. Our results indicate a high resilience of the bacterial community following establishment of the MRF system as its abundance and diversity nearly recovered to levels observed in young stands.

In contrast, bacterial abundance was lower in YRF than in YR at all soil depths, as evidenced by the large reduction in soil microbial respiration in this AFS. This was likely due to the NFS roots exuding specific flavonoids that inhibit a broad range of bacteria (Na et al., 2017). The observed changes mainly resulted from an obvious decline in the abundance of *Actinobacteria*, especially in the rainy season (Table S2), which will have decreased the labile C consumption for latex yield. Another reason is that the NFS cover-induced loss of individual understory plants, but not its diversity, can indirectly contribute to a lower bacterial abundance. Moreover, YRF had no significant effect on bacterial diversity, suggesting a relatively stable and uniform distribution of bacterial communities, which is surprising given that the uncoupling of bacterial abundance and diversity is not commonly observed (de Carvalho et al., 2016). Similarly, bacterial diversity did not respond to forest conversion to rubber and oil palm plantations at a local scale (Lee-Cruz et al., 2013; Kerfahi et al., 2016). Although these studies revealed changes in soil chemistry and hydrology, the consistent bacterial diversity we observed was primarily caused by small variation in near-neutral pH (averaging 6.4). Therefore, after YRF establishment bacterial communities may exhibit a degree of resistance to the changing environment in the short term.

4.3. Bacterial community is vertically stratified

Bacterial abundance and diversity decreased with soil depth, regardless of management type and season, which is similar to the stratified patterns found in other studies (Fierer et al., 2003; López-Mondéjar et al., 2015). Spatial heterogeneity in soil nutrients is primarily responsible for bacterial vertical stratification, representing a strong ecological filter with depth and resulting in many surface-dwelling bacteria being less likely to thrive in deeper soils. Bacterial community structure substantially differed among the three soil depths studied, with the greatest variability observed at 0–5 cm depth (Fig. 5), supporting previous finding that differences in bacterial community among crops and/or sites are more pronounced within the near-surface and intermediate soil depths (Zhang et al., 2017).

Different bacterial taxa exhibited differences in their abundance distribution patterns along the vertical soil profile, which might be related to their life-history strategies. The relative abundances of *Proteobacteria* and *Bacteroidetes* decreased with depth, whereas those of *Acidobacteria* and *Actinobacteria* were greatest in the deepest layer, consistent with patterns observed in previous studies (Eilers et al., 2012; López-Mondéjar et al., 2015). This change resulted in a high ratio of *Proteobacteria* to *Acidobacteria* in the surface layer (Fig. S6), in part reflecting an increase in labile C substrate due to mixed litter decomposition. The increased abundance of *Nitrospirae* with depth may be due to the mediation of nitrite oxidation being positively correlated with organic N, which results from fine root decay (Orr et al., 2015). Fierer et al. (2003) noted that the differentiation of bacterial communities may be affected by additional factors, including the selective transport of bacteria by water and the presence of bacteria in deeper soil derived from original depositional environment over extended periods. However, our sites had the same parent soil material and relatively similar temperature and moisture levels within the top 60 cm of soil (Table 1). Consequently, vertical stratification of nutrient resources predominantly controls bacterial community structure at different soil depths.

4.4. Dominant bacterial groups and key taxa in tropical AFSs

Tropical AFSs of China were dominated by ubiquitous and important bacterial groups, i.e. *Acidobacteria*, *Proteobacteria*, *Actinobacteria* and *Chloroflexi*, similar to those reported from acidic soils in various ecosystems worldwide (Banerjee et al., 2016; de Carvalho et al., 2016; Kerfahi et al., 2016). However, we also observed some interesting patterns in these bacterial communities. For instance, rubber-based AFSs had a greater number of copiotrophic *Proteobacteria* and *Bacteriodetes* than corresponding monocultures at 0–5 cm depth across seasons (Table S2), whereas oligotrophic *Acidobacteria* and *Chloroflexi* were unaffected or negatively affected by AFSs. SOM-degrading *Actinobacteria*, which are typically dominant in stressful soil environments, increased its abundance owing to drought (Ochoa-Hueso et al., 2018). Thus, the observed reduction in *Actinobacteria* abundance in YRF at 60 cm depth, particularly in the rainy season, could be explained by an anoxic environment resulting from high moisture levels (Colombo et al., 2016) or compact soil (Liu et al., 2018). By contrast, a consistent abundance of *Actinobacteria* was observed in MRF, indicating that they are well adapted to this system.

Similar abundance patterns were also evident when bacterial genera were considered, with cellulolytic *Kitasatospora* and *Streptomyces* being present at lower abundance and *Nitrospira* being more ubiquitous in mineral **N**-rich soils of YRF. A higher P content in YRF soils also led to an abundance of phosphate-solubilizing *Bacillus* and *Pseudomonas*. Surprisingly, the abundance of *Bradyrhizobium*, which is a well-known **N**-fixer and denitrifier involved in N cycling, did not change following the NFS introduction, probably due to a mix-up with other **N**-fixing genera in bulk soil. This is congruent with the findings of Lupwayi et al., (2017), who found that the addition of **black** medic (*Medicago lupulina*) has no effect on the abundance of *Bradyrhizobium* despite this genus being most prevalent in the rhizosphere. Simultaneous analyses of the response ratio and the metagenome prediction (Fig. S7) confirm that these changes in key taxa are altering their functional traits, especially in MRF. Therefore, assessing soil microbial functions in AFSs at the genus level seems to be more appropriate.

4.5. Environmental drivers of bacterial communities

Soil pH is considered the best predictor of bacterial community structure and diversity across different spatial scales and soil types (Fierer, 2017). Although there was a high variability in bacterial community composition across the four stands observed here, this was primarily driven by pH at the three depths across seasons (Table S5). For instance, pH was positively correlated with the relative abundances of *Actinobacteria* and *Gemmatimonadetes* and negatively correlated with those of *Acidobacteria* and *Chloroflexi*, supporting previous observations on phylum-level abundance relationships with pH gradients (Sun et al., 2015; Zhang et al., 2017). However, there is no clear relationship between *Proteobacteria* and pH in different soil types (Fierer et al., 2007; Rousk et al., 2010), indicating that this taxon is largely influenced by other factors, particularly C availability, as indicated by its significant correlation with DOC in the present study ($r = 0.53$, $P < 0.001$). These findings confirm that pH integrates variable changes in other soil attributes, thus driving diverse bacterial communities across environmental gradients.

Bacterial diversity was, to a large extent, positively related to pH (Table S4), which supports a quadratic model within the pH range of 3.7–8.5 with a peak at around near-neutral pH (Lauber et al., 2009; Tripathi et al., 2012). This trend is opposite to that observed by Zhou et al. (2017), who found that bacterial diversity was highly negatively correlated with pH in rubber plantations, presumably due to low pH values (< 4.0) promoting oligotrophic abundance (Lee-Cruz et al., 2013). One of the possible reasons for this discrepancy is that intracellular pH for most bacteria is neutral (Fierer and Jackson, 2006), so that if pH falls outside a particular range, it causes direct physiological stress on bacteria by altering competitive outcomes and reducing individual net growth. A stressful environment in the tropics leads to more energy being spent on maintaining pH and more specialized adaptations strategies. It should also be noted that changes in AP and DON concentrations in association with pH functions as a secondary edaphic determinant of bacterial community structure.

5. Conclusions

This study showed that stand age exerted a stronger influence on bacterial communities than management type or season, which was controlled by obvious changes in edaphic factors. Compared to their monocultures, YRF maintained bacterial diversity despite experiencing a decrease in abundance, whereas MRF promoted bacterial diversity with a constant abundance. *Acidobacteria*, *Proteobacteria* and *Actinobacteria* were the dominant bacterial phyla, while *Kitasatospora*, *Streptomyces* and *Nitrospira* were the key genera in tropical AFSs, China. Furthermore, Bacterial communities in AFSs exhibited obvious horizon-specific seasonal variations, which were associated with spatial heterogeneity in soil nutrients, with the surface layer being more nutrient-rich than the intermediate and deep horizons. Soil pH, AP and DON were the major determinants of bacterial community structure. Our findings imply that the establishment of rubber-based AFSs with less-intensive disturbances, particularly in mature stands, could be a good approach for overcoming the detrimental effects of chemical fertilization on bacterial diversity due to the dynamic decomposition of litter and fine roots. Meanwhile, we recommend appropriate liming of acidic soils to maintain the level of pH necessary for optimal bacterial growth. It remains unclear, however, whether the seasonal changes in bacterial communities are directly linked to latex yield. Therefore, although there is a degree of predictability in microbial communities, further research is required to understand the relationship between soil microbes involved in nutrient cycling and AFS productivity under climate change.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.agee.2019.03.015.

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